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Automated Preparative HPLC of Anthocyanins

K.B. HICKS, S.M. SONDEY, D. HARGRAVE, G.M. SAPERS, and A. BILYK
Eastern Regional Research Center, United States Department of Agriculture, Philadelphia, Pennsylvania 19118

Purified anthocyanins obtained from two natural sources — blackberry and cranberry — were isolated by computer-controlled, automated preparative HPLC. The use of the HPLC system in conjunction with a 25 cm × 2.2 cm preparative reversed-phase (C18) column allowed the repetitive, unattended injection and fractionation of milligram to gram quantities of these important, but commercially unavailable, pigments.

INTRODUCTION

Anthocyanins, which are glycosylated polyhydroxy and polymethoxy derivatives of 2-phenylbenzopyrylium salts (Figure 1), are responsible for much of the natural coloration of flowering plants, fruits, and vegetables. Because of the specific distribution, both quantitative and qualitative, of anthocyanins in plants, these compounds serve as highly useful biochemical markers in plant chemotaxonomy (1) and in quality assurance and quality control of foods such as fruit juices (2,3). Various methods exist for the analysis of anthocyanins (for a review, see reference 4), including paper, thin-layer, gas–liquid, and high performance liquid chromatography (HPLC), but quantitation by any of these methods is difficult because of the lack of pure reference standards. Although small amounts (several milligrams) of purified standards are often prepared by preparative paper chromatography and occasionally by small-scale HPLC (1,5,6), no method has been described for rapid, large-scale (multimilligram) preparative HPLC separations of these important natural pigments. The system described here allows such an isolation of blackberry and cranberry pigments and can easily be adapted for other plant pigment isolations.

EXPERIMENTAL

Crude anthocyanins were extracted from berries using the following procedure.

Berries and solvent (85% ethanol, 15% 1.5 *N* HCl), in equal weights (~10 g), were blended for 2 min, then filtered with Celite (Manville, Denver, Colorado) through paper. The extract was carefully evaporated in vacuo at 30 °C and partially purified on columns containing Amberlite CG-50 (Rohm and Haas, Philadelphia, Pennsylvania) (7). After lyophilization, the resulting anthocyanin fraction (stored at −10 °C to prevent degradation) was dissolved in equal amounts of 0.025% HCl in methanol and solvent A (see below) and filtered with a 0.2-μm nylon 66 filter before injection onto the column. Preparative HPLC was performed on a Gilson/Rainin Gradient Auto Prep System composed of two model 303 solvent pumps and a model 302 autoinjector pump (Gilson Medical Electronics, Middleton, Wisconsin), an Apple IIe controller (Apple Computer, Inc., Cupertino, California), a Holochrome 526-nm UV/Vis detector and a model 202 fraction collector (Gilson), and a 25 cm × 2.2 cm Dynamax C18 preparative column (Rainin Instrument Co., Woburn, Massachusetts). Binary gradients comprised 0.1 *M* phosphate buffer, pH 1.5 (solvent A) and acetonitrile (solvent B).

Cranberry anthocyanins were separated under the following conditions. At a flow rate of 12 mL/min, a concave gradient was run, beginning with 11.5% solvent B and finishing with 28% solvent B over a period of 21 min. At 25 min, the gradient returned to initial conditions; autoinjection was performed and a new cycle started at 35 min.

For blackberry anthocyanins, a flow rate of 14 mL/min was set and a concave gradient was run, beginning with 12% solvent B and finishing with 20% solvent B over a period of 20 min. The gradient returned to initial conditions at 21 min, and autoinjection/restart occurred at 30 min.

Pure anthocyanin fractions separated by HPLC were again treated with Amberlite CG-50 as described above and were lyophilized. For analytical HPLC, a 15 cm × 3.9 mm, 5-μm Resolve C18 column (Waters Chromatography Division, Milford, Massachusetts) was used in conjunction with a previously described HPLC system (8). Flow rate was set at 1.5 mL/min, and a binary gradient, initially consisting of 11% solvent B and increasing to 25% solvent B, was run in a 25-min period (curve 7 on gradient controller).

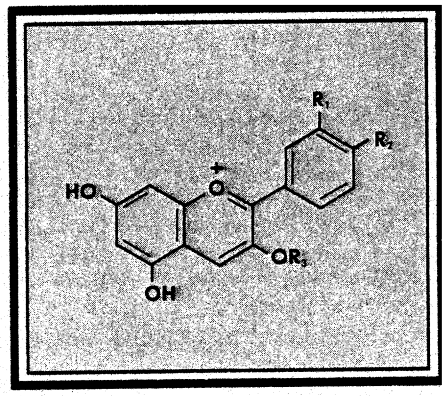

FIGURE 1: Structural features of anthocyanins. Cyanidin-3-glycosides: R_1 = OH, R_2 = OH, R_3 = *O*-glycosyl. Peonidin-3-glycosides: R_1 = OCH_3, R_2 = OH, R_3 = *O*-glycosyl.

All isolated anthocyanins were identified by chromatographic comparison with known standards or, if these were not available, by partial acid hydrolysis (4) and/or UV/Vis spectrophotometry on a model 552 spectrophotometer (Perkin-Elmer Corp., Norwalk, Connecticut), in which case the pigments were dissolved in 0.01% HCl in methanol (4).

RESULTS AND DISCUSSION

Cranberries are known to possess four major anthocyanins: the 3-arabinosides and 3-galactosides of cyanidin and peonidin (Figure 1) (9). Isolation of multimilligram amounts of these pigments was possible with the instrumentation described here. When this system is programmed as diagramed in Figure 2 (using the system software), it allows unattended, repetitive injections of samples, separation of

sample components and "intelligent" peak collection. Injection of 10 mg of Amberlite CG-50 purified extract (Figure 3a) gave a separation with resolution comparable to that given by high resolution analytical columns (Figure 3b). The injection of a single sample using an autoinjector, as is illustrated in Figure 3a, produced the peaks shown in Figure 3c.

The identity of each isolated peak and the amount, purity (based on peak area), and UV/Vis spectral properties of each pigment isolated as a result of ten autoinjections are given in Table I. The purity of each pigment was found to be very high (80%–98%) based on chromatographic peak-area percentages and absorbance ratios (Table I) that are appropriate for cyanidin and peonidin derivatives. For the 3-glucosides of cyanidin and peonidin, absorbance ratios of 60% (Cn-3-glu) and 24% (Pn-3-glu) have been reported for similar purified pigments (4). If pigments of higher purity are desired, they may be repurified with the same system.

Unlike cranberries, blackberries are known to contain one major anthocyanin — cyanidin-3-glucoside — and traces of up to five other pigments (10). Twenty 250-μL (15 mg solids) autoinjections of a blackberry extract (obtained from six varieties) yielded 91 mg of cyanidin-3-glucoside (peak A) at an estimated purity of 98% (Figure 4a). Smaller amounts of three minor pigments were also isolated for qualitative analysis and details of the identity of these peaks will be reported later.

In the work described here, all samples were chromatographed in such a way as to provide high-resolution preparative separations. The combination of small sample size (<20 mg/injection) and efficient column packing (7-μm particle size) makes this possible. More than 200 mg of cyanidin-3-glucoside could be isolated in one continuous 24-h period. Alternatively, larger samples (up to 50 mg) can be injected; and, by "shaving" the poorly resolved peaks, which are caused by column overload, larger quantities of pigments can be isolated more quickly. Because this process led to more rapid column degradation and an isolated product of lesser purity, it was not used extensively.

It is noteworthy that there was no apparent loss of resolution or deterioration of the column over a period of three months of use, even though the acidity of the mobile phase (pH 1.5) was greater than the column manufacturer's recommended value. The dynamic axial compression feature of the column, which serves to remove voids from the column inlet, and nightly flushing of the column with distilled water no doubt contributed to column stability. The cartridge-type column has reusable hardware, and the flow rates and back pressures at which it operates (12–14 mL/min and 60–80 bar pressure in this study) would allow its use on most analytical HPLC systems.

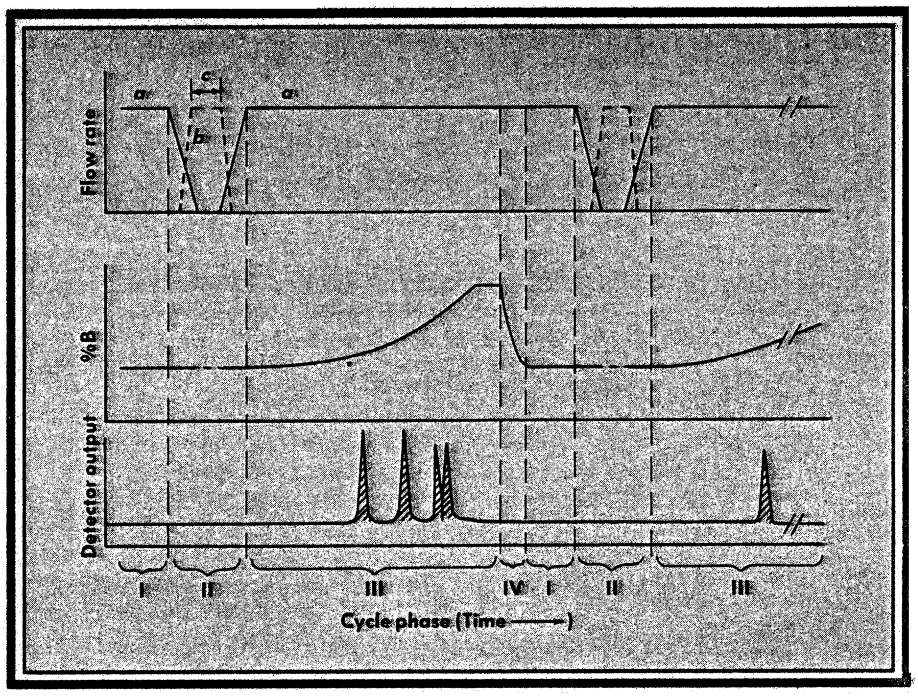

FIGURE 2: Sequences of events during simulated gradient, automated preparative HPLC. The system employs initial solvent conditions during phase I. During phase II, the solvent delivery pumps decrease flow rate *a* as injector pump flow rate *b* is actuated for time *c* necessary to deliver the desired sample volume. During phase III, percent B is modified to produce an appropriate gradient; the peaks are then separated on the column and are automatically collected by the fraction collector. During phase IV, initial conditions are reset for additional cycles. All phases are computer controlled.

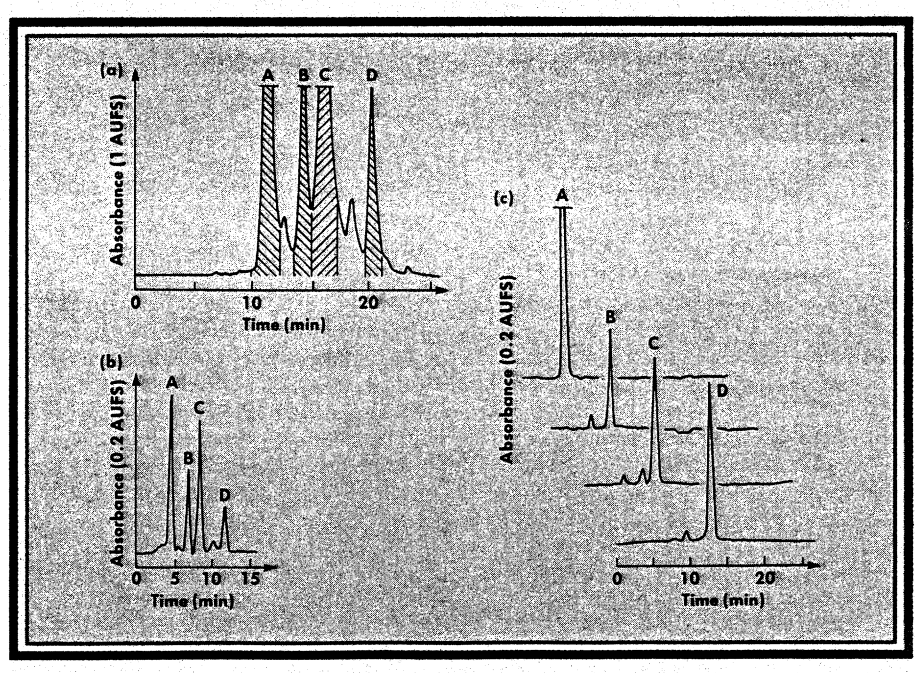

FIGURE 3: Analytical and preparative HPLC separations of pigments from cranberry extract. (a) Single autoinjection of 10 mg (200 μL) of crude cranberry anthocyanins; crosshatched areas represent peaks automatically collected by fraction collector. (b) Analytical HPLC; a 5-μL (<1 μg) injection of cranberry extract. (c) Analytical HPLC separation of fractions collected in (a). All detection at 546 nm; other conditions listed in the Experimental section. Peaks: A = Cn-3-gal, B = Cn-3-ara, C = Pn-3-gal, D = Pn-3-ara.

TABLE I: PROPERTIES OF CRANBERRY ANTHOCYANINS ISOLATED BY PREPARATIVE HPLC FROM TEN AUTOINJECTIONS (FIGURE 3)

Peak	Isolated Weight (mg)	Purity (%)*	$(A_{UV\,max}/A_{Vis\,max})$** (%)	$(A_{440}/A_{Vis\,max})$† (%)
A	20	98	58	25
B	7	81	58	22
C	24	85	63	24
D	10	88	62	24

*purity estimated on an area percent basis from analytical chromatogram
**ratio of absorbance values at UV maximum wavelength (280 nm) to that at visible maximum wavelength (527 nm)
†ratio of absorbance values at 440 nm to that at visible maximum wavelength

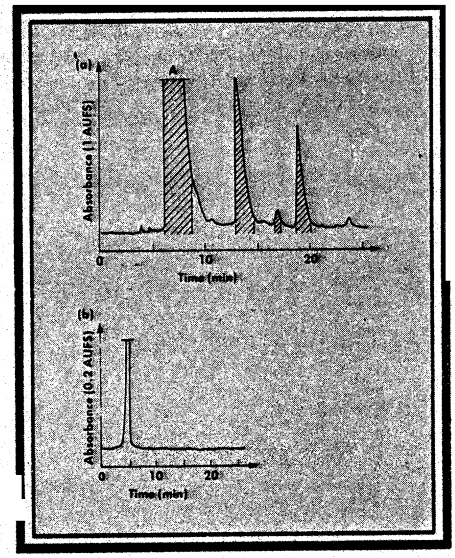

FIGURE 4: HPLC separation of blackberry anthocyanins. (a) Automatic preparative HPLC using 15 mg (250 μL) of crude blackberry anthocyanins. (b) Analysis of peak A (cyanidin-3-glucoside) by analytical HPLC.

In summary, gradient automated preparative HPLC can be used for the isolation of purified anthocyanins. The availability of such pigments will allow the quantification of anthocyanins in natural matrices and will be useful in food-regulatory, plant-patent-protection, and plant biochemical applications.

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